spectrometry and chip hybridization. The invention is illustrated using electrophoretic separation for the analysis of the products of the cleavage reactions. However, it is noted that the resolution of the cleavage products is not limited to electrophoresis. Electrophoresis is chosen to illustrate the method of the invention because electrophoresis is widely practiced in the art and is easily accessible to the average practioner.

The probe and invader oligonucleotides may contain a label to aid in their detection following the cleavage reaction. The label may be a radioisotope (*e.g.*, a ^{32}P or ^{35}S-labelled nucleotide) placed at either the 5' or 3' end of the oligonucleotide or alternatively, the label may be distributed throughout the oligonucleotide (*i.e.*, a uniformly labelled oligonucleotide). The label may be a nonisotopic detectable moiety, such as a fluorophore, which can be detected directly,or a reactive group which permits specific recognition by a secondary agent. For example, biotinylated oligonucleotides may be detected by probing with a streptavidin molecule which is coupled to an indicator (*e.g.*, alkaline phosphatase or a fluorophore) or a hapten such as dioxigenin may be detected using a specific antibody coupled to a similar indicator.

Optimization Of Reaction Conditions

The invader-directed cleavage reaction is useful to detect the presence of specific nucleic acids. In addition to the considerations listed above for the selection and design of the invader and probe oligonucleotides, the conditions under which the reaction is to be performed may be optimized for detection of a specific target sequence.

One objective in optimizing the invader-directed cleavage assay is to allow specific detection of the fewest copies of a target nucleic acid. To achieve this end, it is desirable that the combined elements of the reaction interact with the maximum efficiency, so that the rate of the reaction (*e.g.*, the number of cleavage events per minute) is maximized. Elements contributing to the overall efficiency of the reaction include the rate of hybridization, the rate of cleavage, and the efficiency of the release of the cleaved probe.

The rate of cleavage will be a function of the cleavage means chosen, and may be made optimal according to the manufacturer's instructions when using commercial preparations of enzymes or as described in the examples herein. The other elements (rate of hybridization, efficiency of release) depend upon the execution of the reaction, and optimization of these elements is discussed below.

Three elements of the cleavage reaction that significantly affect the rate of nucleic acid hybridization are the concentration of the nucleic acids, the temperature at which the cleavage reaction is performed and the concentration of salts and/or other charge-shielding ions in the reaction solution.

The concentrations at which oligonucleotide probes are used in assays of this type are well known in the art, and are discussed above. One example of a common approach to optimizing an oligonucleotide concentration is to choose a starting amount of oligonucleotide for pilot tests; 0.01 to 2 μM is a concentration range used in many oligonucleotide-based assays. When initial cleavage reactions are performed, the following questions may be asked of the data: Is the reaction performed in the absence of the target nucleic acid substantially free of the cleavage product?; Is the site of cleavage specifically shifted in accordance with the design of the invader oligonucleotide?; Is the specific cleavage product easily detected in the presence of the uncleaved probe (or is the amount of uncut material overwhelming the chosen visualization method)?

A negative answer to any of these questions would suggest that the probe concentration is too high, and that a set of reactions using serial dilutions of the probe should be performed until the appropriate amount is identified. Once identified for a given target nucleic acid in a give sample type (*e.g.*, purified genomic DNA, body fluid extract, lysed bacterial extract), it should not need to be re-optimized. The sample type is important because the complexity of the material present may influence the probe optimum.

Conversely, if the chosen initial probe concentration is too low, the reaction may be slow, due to inefficient hybridization. Tests with increasing quantities of the probe will identify the point at which the concentration exceeds the optimum. Since

the hybridization will be facilitated by excess of probe, it is desirable, but not required, that the reaction be performed using probe concentrations just below this point.

The concentration of invader oligonucleotide can be chosen based on the design considerations discussed above. In a preferred embodiment, the invader oligonucleotide is in excess of the probe oligonucleotide. In a particularly preferred embodiment, the invader is approximately 10-fold more abundant than the probe.

Temperature is also an important factor in the hybridization of oligonucleotides. The range of temperature tested will depend in large part, on the design of the oligonucleotides, as discussed above. In a preferred embodiment, the reactions are performed at temperatures slightly below the T_m of the least stable oligonucleotide in the reaction. Melting temperatures for the oligonucleotides and for their component regions (X, Y and Z, Figure 29), can be estimated through the use of computer software or, for a more rough approximation, by assigning the value of 2°C per A-T basepair, and 4°C per G-C basepair, and taking the sum across an expanse of nucleic acid. The latter method may be used for oligonucleotides of approximately 10-30 nucleotides in length. Because even computer prediction of the T_m of a nucleic acid is only an approximation, the reaction temperatures chosen for initial tests should bracket the calculated T_m. While optimizations are not limited to this, 5°C increments are convenient test intervals in these optimization assays.

When temperatures are tested, the results can be analyzed for specificity (the first two of the questions listed above) in the same way as for the oligonucleotide concentration determinations. Non-specific cleavage (*i.e.*, cleavage of the probe at many or all positions along its length) would indicate non-specific interactions between the probe and the sample material, and would suggest that a higher temperature should be employed. Conversely, little or no cleavage would suggest that even the intended hybridization is being prevented, and would suggest the use of lower temperatures. By testing several temperatures, it is possible to identify an approximate temperature optimum, at which the rate of specific cleavage of the probe is highest. If the oligonucleotides have been designed as described above, the T_m of the Z-region of the probe oligonucleotide should be below this temperature, so that turnover is assured.